The effect of two Terpenoids, Ursolic acid and Oleanolic acid on epidermal permeability barrier and simultaneously on dermal functions

Suk Won Lim[1], Sung Won Jung[1], Sung Ku Ahn[3], Bora Kim[1], Hee Chang Ryoo[1], and Seung Hun Lee[2]

[1]R & D Center of Skin Science & Cosmetics, ENPRANI Co., Ltd, Inchon, 400-103, Korea
[2]Dept. of Dermatology, Dermatology, Yonsei Univ. college of Medicine, Seoul, 135-720, Korea
[3]Dept. of Dermatology, Dermatology, Yonsei Univ. college of Medicine, Wonju, 220-701, Korea

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Summary

Ursolic acid (UA) and Oleanolic acid (ONA), known as urson, micromerol and malol, are pentacyclic triterpenoid compounds which naturally occur in a large number of vegetarian foods, medicinal herbs, and plants. They may occur in their free acid form or as aglycones for triterpenoid saponins, which are comprised of a triterpenoid aglycone, linked to one or more sugar moieties. Therefore UA and ONA are similar in pharmacological activity. Lately scientific research, which led to the identification of UA and ONA, revealed that several pharmacological effects, such as antitumor, hepato-protective, anti-inflammatory, anticarcinogenic, antimicrobial, and anti-hyperlipidemic could be attributed to UA and ONA. Here, we introduced the effect of UA and ONA on acutely barrier disrupted and normal hairless mouse skin. To evaluate the effects of UA and ONA on epidermal permeability barrier recovery, both flanks of 8-12 week-old hairless mice were topically treated with either 0.01-0.1 mg/ml UA or 0.1-1 mg/ml ONA after tape stripping, and TEWL (Transepidermal water loss) was measured. The recovery rate increased in those UA or ONA treated groups (0.1 mg/ml UA and 0.5 mg/ml ONA) at 6 h more than 20% compared to vehicle treated group ($p<0.05$). Here, we introduced the effects of UA and ONA on acute barrier disruption and normal epidermal permeability barrier function. For verifying the effects of UA and ONA on normal epidermal barrier, hydration and TEWL were measured for 1 and 3 weeks after UA and ONA applications (2mg/ml per day). We also investigated the features of epidermis and dermis using electron microscopy (EM) and light microscopy (LM). Both samples increased hydration compared to vehicle group from 1 week without TEWL

alteration ($p<0.005$). EM examination using RuO4 and OsO4 fixation revealed that secretion and numbers of lamellar bodies and complete formation of lipid bilayers were most prominent (ONA≥ UA>Vehicle). LM finding showed that thickness of stratum corneum (SC) was slightly increased and especially epidermal thickening and flattening was observed (UA>ONA>Veh). We also observed that UA and ONA stimulate epidermal keratinocyte differentiation via PPAR α. Protein expression of involucrin, loricrin, and filaggrin increased at least 2 and 3 fold in HaCaT cells treated with either ONA (10 μM) or UA (10 μM) for 24h respectively. This result suggested that the UA and ONA can improve epidermal permeability barrier function and induce the epidermal keratinocyte differentiation via PPAR α. Using Masson-trichrome and elastic fiber staining, we observed collagen thickening and elastic fiber elongation by UA and ONA treatments. *In vitro* results of collagen and elastin synthesis and elastase inhibitory activity measurements were also confirmed *in vivo* findings. These data suggested that the effects of UA and ONA related to not only epidermal permeability barrier functions but also dermal collagen and elastic fiber synthesis. Taken together, UA and ONA can be relevant candidates to improve epidermal and dermal functions and pertinent agents for cosmeseutical applications.

Introduction

The skin is the largest organ in the body in terms of coverage by surface area [76]. It is waterproof and maintains an ideal environment in the body in spite of varying external and core temperatures.

The skin is made up of two layers – an outer epidermis and an inner dermis. The epidermal permeability barrier plays a crucial role in human physical, chemical, and biological cutaneous functions. This barrier function resides in the outermost layer of the epidermis, stratum corneum (SC), which consists of two major structural components, corneocytes and intercorneocyte lipids. Intercorneocyte lipid in the SC provides a bi-directional barrier to water and electrolyte movement. It is mediated by lamellar bilayers, enriched in cholesterol, free fatty acids and ceramides [1]. The SC mediates most of the protective functions of the skin against excessive loss of body water, as well as provides a mechanical shield against external insults and prevents the invasion of foreign substances and pathogens [2-3].

The epidermis is an active site of lipid synthesis. Excepts the essential fatty acids that are obtained from the circulation, keratinocytes are able to synthesize all lipid species necessary for the proper functioning of the epidermis. During the migration of keratinocytes from the basal layer to the SC, a considerable change in lipid composition takes place [1]. These changes contain a progressive depletion of phospholipids and glycosphingolipids, concomitant with an increase of ceramides, cholesterol, free fatty acid, and small amounts of cholesterol sulfate and cholesterol esters [4]. These lipids are delivered to the intercorneocyte spaces as a mixture of precursors by the secretion of lamellar body (LB). Following their secretion, the lipid precursors are metabolized within the extracellular spaces by colocalized, LB-derived hydrolytic enzymes into hydrophobic, lamellar basic unit structures, which mediate barrier function [5]. Prior studies suggested that the skin barrier to water loss was attributable to the SC lipids [2,6], and the impaired barrier function is indicated by increased transepidermal water loss (TEWL) and diminished water-holding properties. Imokawa *et al.* [7] reported that intercellular lipids, especially ceramide, play a critical role in water-holding properties of the SC. The barrier abnormality correlates with reduction in these stratum corneum lipids, along with a selective reduction in one or two of three main intercellular lipid species, cholesterol, free fatty acid, ceramide. Even though ceramide itself was known be effective components on skin barrier function, using only ceramide on skin was not effective. In this study, we would like to answer whether improvement of skin barrier can be the solution for cutaneous dysfunction and sustain normal skin barrier function.

The skin is composed of two types of connective tissues such as collagen (a strong white

fiber) and elastin (a thin yellow elastic fiber). Collagen is the major dermis structural protein. Manufactured by fibroblasts, the arrangement of collagen fibrils into small groups of fibers which then come together in thin, wavy, fiber bundles. These bundles are then arranged into a mat-like pattern resulting in great tensile strength. Elastic fibers composed mostly of the protein elastin, found in a network in the dermis. These fibers maintain normal skin tension, and give it the ability to snap back after stretching. Alterations in the elastic fiber network are responsible for looseness and sagging of aged skin. Repeated exposure of the skin to UV radiation damages these fibers. Gradually, the amount of collagen present in the skin is decreased. Concurrently, the amount of elastic fibers increases, however, the elastic fibers loose some of their elasticity. Collagen and elastic fibers are part of the extracellular complex of macromolecules in the connective tissue. Changes in these fibers are thought to play an important role in aging as well as in several pathological conditions. Tsukahara K *et al.* [75] previously reported that chronic UVB irradiation causes wrinkle formation, decreases skin elasticity, and damages/curls dermal elastic fibers. Those UVB-induced wrinkles can be improved by treatment with retinoic acid or with a CO_2 laser, which results in a recovery of skin elasticity and a repair of elastic fiber linearity. We showed further that topical application of N-phenetyl-leucyl-tryptophane, an agent that specifically inhibits fibroblast-derived elastase, immediately after UVB irradiation inhibited UVB-induced wrinkle formation, maintained skin elasticity, and inhibited changes in the three-dimensional structure of dermal elastic fibers in a dose-dependent manner. In this study, the effect of an extract of *Sanguisorba officinalis L.*, which also inhibits fibroblast-derived elastase, was evaluated for possible inhibition of UVB induced wrinkle formation, maintenance of skin elasticity, and prevention of damage to the 3-dimensional structure of dermal elastic fibers. Hind limb skins of 3-week-old Sprague–Dawley rats were irradiated with UVB at a suberythemal dose 3 times a week for 6 weeks. Simultaneously, an extract of *Sanguisorba officinalis L.* (at 0.2% (v/v) or 1% (v/v)) was topically applied 5 times per week immediately following each UVB irradiation and 1 d later. The extract of *Sanguisorba officinalis L.* inhibited wrinkle formation, maintained skin elasticity, and inhibited the decrease of dermal elastic fiber linearity in the rat hind limb skin in a dose-dependent manner. We have confirmed that the inhibition of elastase activity in fibroblasts immediately after UVB irradiation using an extract of *Sanguisorba officinalis L.* prevents chronic photodamage following UVB irradiation. When you are young, your skin is thick and elastic-high in Collagen and Elastin fibers. As one grows older, the body slows down the production of the essential ingredients it requires to maintain its youthful appearance and elasticity. As a result, the skin becomes thinner and more susceptible to wrinkles, sagging skin, bruising and tearing of the outer layer.

Ursolic acid (UA) and Oleanolic acid (ONA), a pentacyclic triterpene acid, are isolated from various medicinal plants, such as *Eriobotrya japonica, Rosmarinus officinalis*, and *Glechoma*

hederaceae [8-10]. Some of UA [(3β-3-Hydroxyurs-12-en-28-oic acid) rarely occurs without its isomer ONA [(3β)-3-Hydroxyolean-12-en-28-oic acid]. They may occur in their free acid form or as aglycones for triterpenoid saponins, which are comprised of a triterpenoid aglycone, linked to one or more sugar moieties. Even the different sites of the methyl group of the E loop, they have similar molecular structure and pharmacological activity [11-13]. There is a growing interest in the elucidation of the biological roles of triterpenoid compounds, the major components of many traditional medicinal plants [14,15], in terms of hepatoprotectory, analgesic, antitumor, anti-inflammatory, and immunomodulatory effects.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belong to the steroids nuclear receptor superfamily. Three different PPAR have been identified in mammals [16-18]. PPAR α is expressed in many tissues, including the heart, kidney, liver, and epidermis, that it is an important regulator of lipid metabolism [17,18]. With epidermal differentiation, the expression of PPAR α increases and stimulations of PPAR α stimulates the synthesis of cholesterol and ceramides in keratinocytes [19,20]. Recent work has emphasized the importance of PPAR α in keratinocyte differentiation and epidermal permeability barrier. Administration of PPAR α agonist was found to stimulate rodent keratinocyte differentiation *ex vivo* [21] and to the accelerate development of the fetal epidermal permeability barrier [22,23]. PPAR α activators profoundly influenced lipid metabolism in reconstructed epidermis,[20] and recently it was demonstrated that topical treatment with PPAR ligands promoted differentiation and reversed induced hyperproliferation of murine epidermis [24,25]. Moreover, in two separate models of hyperproliferative epidermis, essential fatty acid deficiency and repeated permeability barrier disruption, topical treatment with PPAR α activators restores epidermal homeostasis [23].

The role of PPAR α in human epidermal keratinocyte differentiation and SC lipid barrier formation has been suggested which based on the ability of potent PPAR α activators, Clofibrate and WY-14643, to increase the expression of cornified envelope (CE) associated proteins such as involucrin, filaggrin, loricrin, keratin 1, 10 and transglutaminase. The CE that is formed during the terminal differentiation of keratinocytes is a specialized structure, which forms a structurally and functionally complete epidermal permeability barrier.

Materials and Methods

Animals

Adult hairless mice purchased from the animal laboratory of Yonsei University (Skh-1), were fed a standard mouse diet and water *ad libitum*. 8 to 12 weeks of hairless mice were used in the study.

Cell Culture

HaCaT were obtained from American Type Culture Collection (ATCC; Rockville, U.S.A.). The cell wall maintained in DMEM supplemented with 10% FBS (GibcoBRL Grand Island, NY) (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B; GibcoBRL Grand Island, NY) in CO_2 incubator.

Reagents

Polyethylene glycol (PEG) and ethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Cellophane tapes (3M, scotch tape) were used for barrier disruption. PPAR α antibodies were purchased from Santa Cruz Biotechnology and cornified envelope proteins involucrin, loricrin, and filaggrin purchased from BabCO (Covance, Berkley, CA). Protein quantity was determined using the Commasie Plus Protein Assay Reagent (PIERCE). All experiment reagents were used as the analytic grade.

Barrier Disruption and TEWL Measurement

Barrier disruption was achieved by repeated applications of cellophane tape (Scotch Crystal Clear Tape, 3M, St. Paul, MN) on mouse flank skin. The procedure was completed when transepidermal water loss (TEWL) levels achieved 4 mg/cm^2/h. TEWL was assessed using a Tewameter (TM210; Courage and Khazaka, Cologne, Germany). Immediately after barrier disruption, 250 μl of UA and ONA were applied to the one side of flank and vehicle (PEG:EtOH = 7:3) was applied to the other side of flank as a control. TEWL was measured before, immediately after, and at 3h, 6h, and 24h after barrier disruption. In each animal the percentage recovery was calculated using the following formula: [1- (TEWL at indicated time – baseline TEWL) / (TEWL immediately after treatment – baseline TEWL)] × 100%. All data were compared with data from simultaneously studied controls.

Hydration and TEWL Measurement on Normal Skin

Without barrier disruption, 250 μl of UA and ONA (2 mg/ml each) were applied to the normal hairless mice skin and vehicle (PEG:EtOH = 7:3) were applied as a control. Repeated application of UA and ONA on mouse flank skin was continued for 1 and 3 weeks to determine the effects of UA and ONA on normal epidermal barrier function. Skin hydration was assessed by arbitrary capacitance unit values (a. u.) with a Corneometer (CM 825; Courage & Khazaka). TEWL was measured for substantiating the side effects of UA and ONA on epidermal permeability barrier function. The existing guidelines for assessment of these parameters were followed [26,26].

Light Microscopic Examination

Hematoxylin and Eosin (H&E) Stain

All biopsies for light microscopy were fixed in 10% formalin. After routine processing and embedding in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin. We evaluated changes in the epidermal thickness and smoothness under a microscope.

Collagen Staining

After deparaffinized and rehydrate slides to ddH_2O, place slides in Bouin's fixative overnight at RT. Then, wash the sample slides in running water until sections are colorless and rinse again. After stain in Weigert's Iron hematoxylin for 10 min then wash and rinse, stain in Biebrich scarlet-acid Fuchsin solution for 15min. Rinse in deionized/distilled water. Place the slides in phosphomolybdic/phosphotungstic acid solution for 10-15 min. Stain sections in Aniline Blue solution for 10-20 min. Rinse slides briefly in distilled water, place slides in 1% acetic acid solution for 3-5 min to discard solution. Dehydrate to xylene, clear the EtOH and covership slides.

Elastin Fiber Staining

After deparaffinized, rinse slides in alcohol and ddH_2O. Place the slides in acid potassium permanganate (0.5% potassium permanganate in 3% sulphuric acid) for 5 min, then rinse in distilled water. Decolorized in 1% oxalic acid for 2 min, then wash the slides in destiled water

again. After rinse in 95% alcohol, place the slides in Miller's stain in a closed container for 1-3 hours then rinse again in 95% alcohol and water. Stain with Weigert's haematoxylin for 10 min. Wash the slides in tap water, differentiate in acid alcohol (1% hydrochloric acid in 70% alcohol for 5 sec then immediately wash the slides in tap water for 5 min. Stain with van Gieson's stain for 1 min and rinse quickly in distilled water. After blot dry, rinse very quickly in alcohol, clear and mount.

Collagen Protein Measurement

Total soluble collagens were measured in culture supernatants by the Sircol™ assay (Biocolor, Belfast. N. Ireland). Sirus Red reagent (1 ml) was added to 150 μl test sample and mixed for 30 min at RT. The collagen-dye complex was precipitated by centrifugation at 16,000 X g for 5 min; washed twice with 0.5 ml of methanol-free ethanol and dissolved in 1 ml 0.5M NaOH; the absorbance was measured at 540 nm. The calibration curve was set up on the basis of collagen standard provided by the manufacture. The assay was performed in triplicate.

Elastin Protein Measurement

Total elastin contents were calculated with a FASTIN™ assay (Biocolor, Belfast, Ireland) in culture supernatant. Fastin Dye Reagent (1 ml) and 90% saturated ammonium sulfate was added to 100 μl test sample and placed for overnight in cold room. The elastin-dye complex was precipitated by centrifugation at 10,000 X g for 10 min; washed twice with 0.5 ml of methanol-free ethanol and dissolved in 1 ml Fastin Dissociation Reagent; the absorbance was measured at 513 nm. The calibration curve was set up on the basis of a-elastin standard provided by the manufacture. The assay was performed in triplicate.

Electron Microscopic Examination

The biopsy specimens were immediately cut into 1 mm pieces and placed in a solution of 2% glutaraldehyde, 2% paraformaldehyde, 0.06% calcium chloride, 0.1 M sodium cacodylate buffer pH 7.4. Specimens were left at room temperature for 1 h and then refrigerated at 4°C for 18-24 h. Each specimen was then rinsed with 0.1 M cacodylate buffer three times at 40 min intervals. For RuO_4 post-fixation the specimens were post-fixed in 0.25% RuO_4 (Polysciences), 0.1 M cacodylate buffer for 45 min at room temperature in the dark. Then the specimens were split, postfixed in 0.2% ruthenium tetroxide (RuO_4) to evaluate intercellular multi-layers and postfixed in 1% osmium tetroxide (OsO_4) to examine the SC layers, routinely processed and embedded in Epon-epoxy resin mixtures. Ultrathin section (60-80nm) were double-stained with uranyl acetate

and lead citrate, and then examined with an electron microscope [28,29].

Elastase Inhibitory Effect Test

Porcine pancreatic elastase (PPE: Sigma) was assayed spectrophotometically by the method of James *et al* [73], using N-Succnyl-$(Ala)_3$-p-nitroanilide (Sigma, St. Louis, USA) as the substrate and monitoring the release of p-nitroaniline for 20 min at 37°C. The amount of p-nitroaniline was determined by measuring at 400 nm. The reaction mixture contained 0.2 M Tris-HCl buffer (pH 8.0), 3.5 unit/ml elastase, 0.25 mM N-Succinyl-$(Ala)_3$-p-nitroanilide (Sigma, St. Louis, USA) as substrate and test sample with dissolved. Blanks contained all the components except the enzyme. Inhibition (%) = (ODexp. – ODcon.) / ODexp. X 100

Protein Extraction and Immunoblot Analysis

Cells (2×10^6/dish) were lysed with lysis buffer (6.25 mM Tris-HCl pH 6.8, 20% Glycerol, 2% (W/V) SDS, 5% beta-mercaptoethanol) at RT. After boiling 100°C for 10 min, cells were centrifuge 10000 rpm for 2 min and supernatants were collected. The protein content measured by Coomassie Plus Protein Assay Reagent (PIERCE). Equal amounts of cell lysate protein were resolved by SDS-polyacrylamide gel electrophoresis (10% running gel) and transferred onto nitrocellulose membrane (Schleicher&Schuell). The membrane was incubated with blocking solution (5% skim milk in Tris-buffered saline containing Tween 20; TBS-T) at RT for 1 h, and incubated with primary antibodies for 2 h. After washing (TBS-T), incubated with secondary antibodies (1 h) then the membrane was washed again with Tris-buffered saline (0.15 M NaCl, 0.02 M Tris-HCl pH 7.6, 0.05% Tween 20) solution. Signals were detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The primary antibodies were used at dilution 1:250 to 1:2000. The secondary antibodies were anti-mouse, anti-rabbit or anti-goat IgG conjugated to horseradish peroxidase (dilution 1:2000).

Statistical Analysis

Student's paired *t* test was used for statistical analyses. $P < 0.05$ was considered significant.

Results

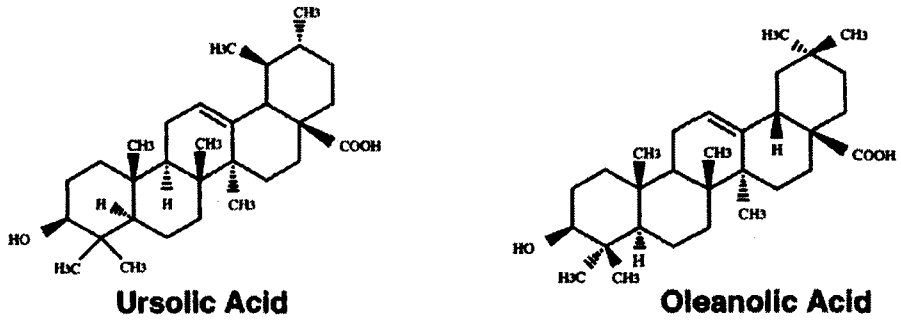
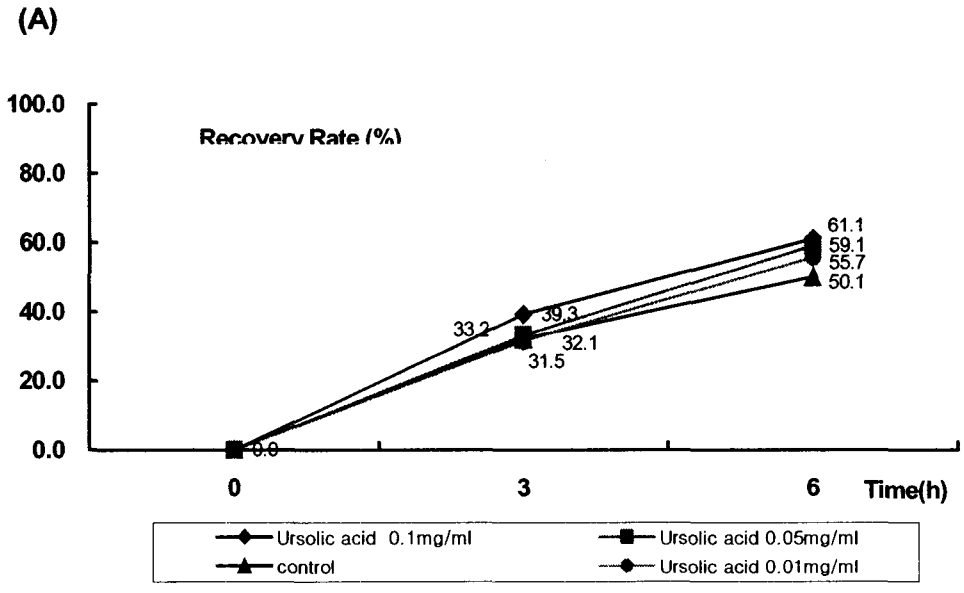


Fig. 1: Molecular structure of UA and ONA

Barrier recovery after barrier perturbation

To evaluate whether the UA and ONA could influence barrier recovery after acute barrier disruption, each molecules were applied to barrier disrupted hairless mice skin. Tape stripping removes not only intercellular lipids but also the cellular components of the SC. As seen in Fig 2, following mechanical disruption of the barrier by tape stripping, application of UA and ONA significantly accelerate barrier repair function. 0.1 mg/ml UA (A) and 0.5 mg/ml ONA (B) treated groups showed the best recovery rate. The recovery rate increased in these groups at 6 h more than 20% compared to vehicle group ($p<0.05$) (n=10). TEWL level was also measured the till 24h for checking side effects and toxicity of UA and ONA applications. However there were no TEWL changes observed compared with vehicle treated group (data not shown).



(B)

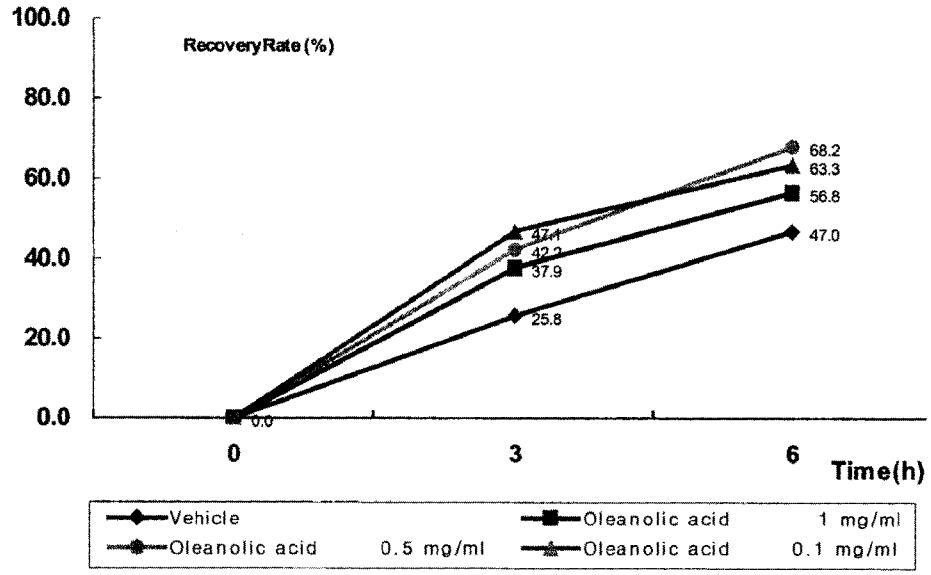
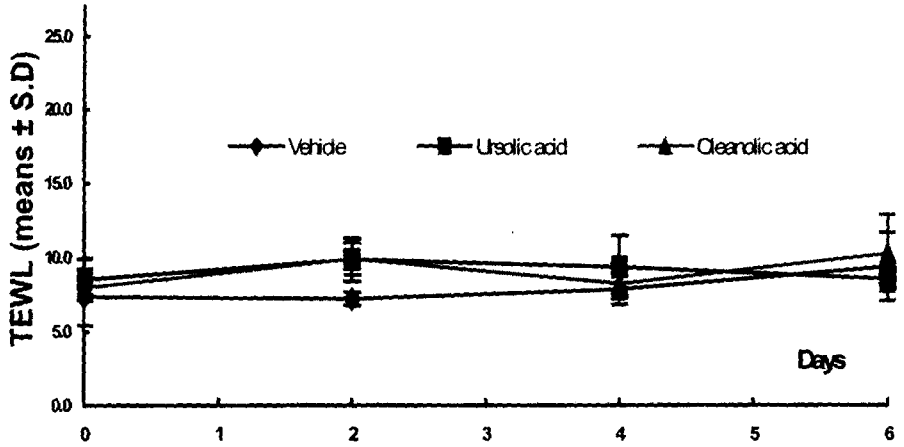


Fig. 2: Effects of UA and ONA on acute barrier disruption: After tape stripping, each hairless mice treated with various UA (A) and ONA (B) measured the TEWL level in each time point. The values are mean ± sd, n=10 and statistical analysis was by student's *t*-test; $p<0.05$ at 6 h cf. vehicle treatment.

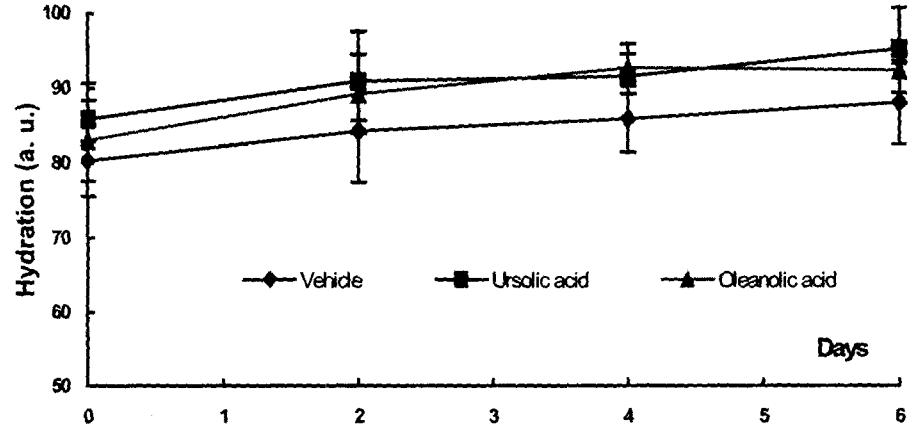
Skin hydration and TEWL in normal Skin Barrier

SC hydration, represented by skin capacitance value, was changed by topical UA and ONA application. Without acute barrier disruption, each group (n=10) was treated with ONA and UA for 1 week (Fig. 3 A, B) and 3 (Fig. 3 C, D) weeks, then the skin capacitance and TEWL were measured. Both groups showed considerable increasing hydration level compared to vehicle treated group from 1 week ($p<0.05$) without significant TEWL alteration. The skin capacitance values are given in arbitrary capacitance units (a. u.) as means ± S.D. (n=20).

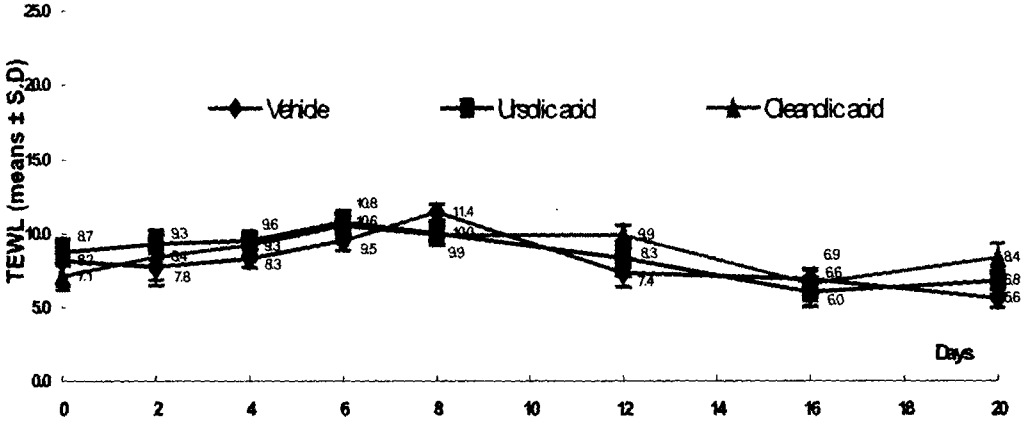
(A)


TEWL (means ± S.D)
25.0
20.0
15.0
10.0
5.0
0.0
Vehicle
Ursolic acid
Oleanolic acid
Days
0
1
2
3
4
5
6

(B)


Hydration (a. u.)
100
90
80
70
60
50
Vehicle
Ursolic acid
Oleanolic acid
Days
0
1
2
3
4
5
6

(C)


TEWL (means ± S.D)
25.0
20.0
15.0
10.0
5.0
0.0
Vehicle
Ursolic acid
Oleanolic acid
Days
0
2
4
6
8
10
12
14
16
18
20

(D)

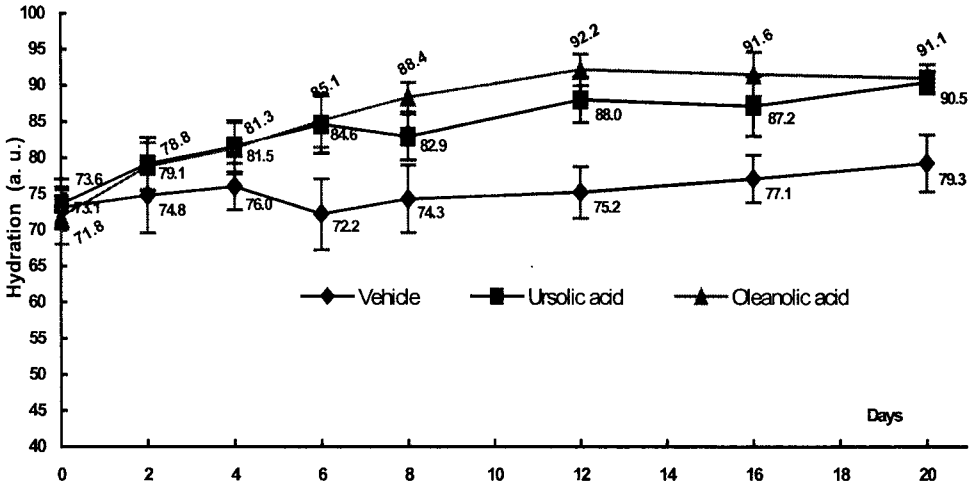


Fig. 3: Effects of UA and ONA on TEWL level and Hydration. Without acute barrier disruption, each hairless mouse treated with 250 μl of UA (2 mg/ml) and ONA (2 mg/ml) for 1week (A, B) and 3weeks (C, D). Measured the TEWL levels and hydration (arbitrary capacitance units) in each time point. The values are mean ± sd, n=20 and statistical analysis was by student's *t*-test; (B) UA treated group from 2d to 6d = $p<0.05$ and ONA treated group at 4d = $p<0.05$ cf. vehicle treatment. (D) UA treated group from 2d to 4d = $p<0.05$, from 6d to 20d = $p<0.0005$ and ONA treated group 4d = $p<0.05$, and from 6d to 20d = $p<0.0005$.

Microscopic examinations of epidermis

Epidermal thickness and smoothness were observed under light microscopy (LM) for substantiating morphologic changes with the UA and ONA applications ($p<0.05$). LM findings demonstrated slightly increased of SC thickness, significant epidermal thickening (UA 21.5% and ONA 14.5% increased, table 1) and flattening were observed (UA>ONA>vehicle, Fig. 4). Morphological changes of the SC layers and intercellular lipid bilayers in normal epidermal barrier with long-term (1, 3 weeks) treatment of the UA and ONA were examined using the RuO_4 postfixation with an electron microscope. There were no remarkable differences in the number of SC layers between UA and ONA application. However, the layers of SC in the skin with the UA and ONA were slightly increased as compared with vehicle (Fig. 5), it also showed well-conserved intercellular lipid multilayers (arrow) and intact lacuna (L) in UA and ONA treated groups as like vehicle group even after 3 weeks application (Fig. 5). It is suggested that appropriately concentrated UA and ONA treated groups have no defects for epidermal permeability barrier function. The number of LB was slightly increased with UA and ONA application compared with vehicle. Increased LB production was evidenced by rising in the quantities of newly secreted extracellular lamellae in skin treated with UA and ONA (data not

shown).

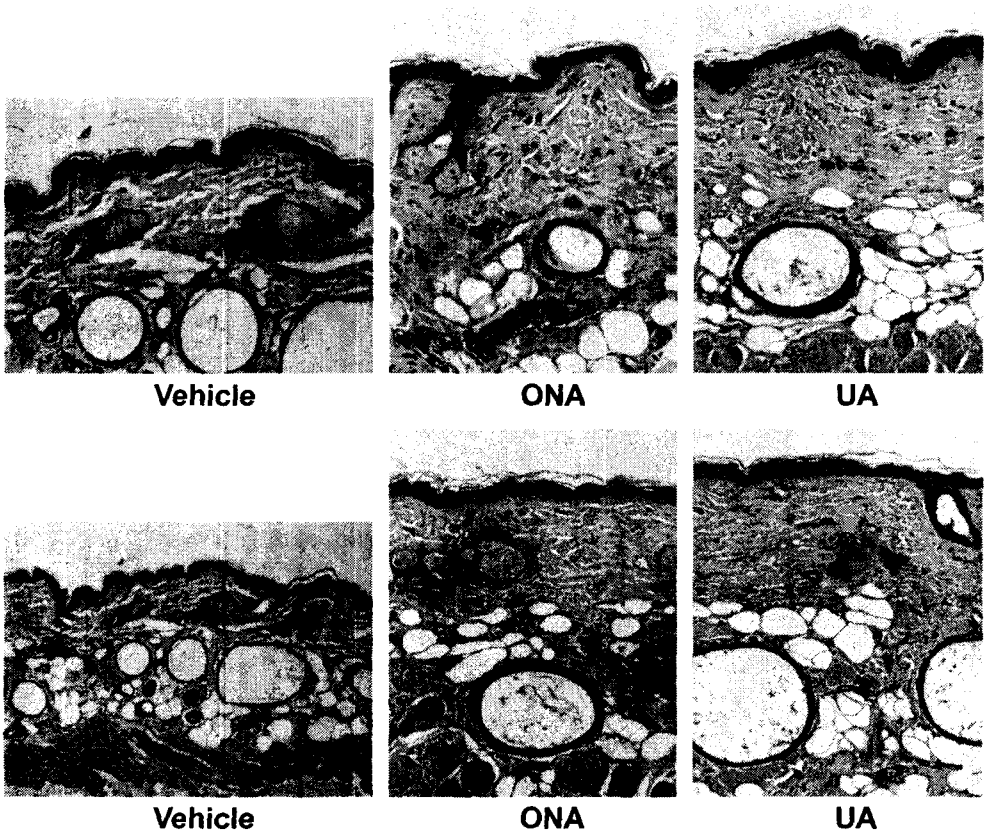


Fig. 4: Histological findings of vehicle, UA, and ONA treated hairless mice skin. Repeated UA ONA, and vehicle treated for 1 week (upper) and 3 weeks (lower). All specimens showed normal-looking appearance of epidermis and dermis. LM findings showed epidermal thickness and flattening were observed (UA>ONA>vehicle, H&E stain, X200).

<Table 1>

	Control	*ONA*	*UA*
1 week	**41.87**	**45.38**	**49.80**
	100%	**108.4%**	**118.9%**
3 weeks	**42.89**	**49.11**	**52.12**
	100%	**114.5%**	**121.5%**

Each group $N > 30$ $p < 0.05$

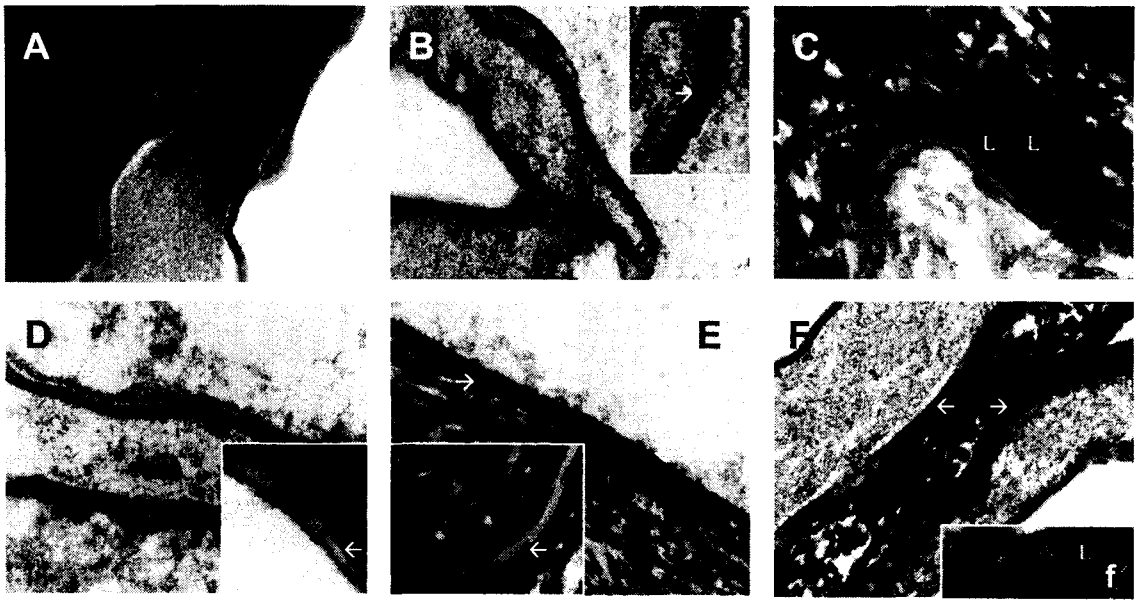


Fig. 5: Electron microscopic findings of SC interstices of 1 (A~C) and 3 (D~F) weeks UA and ONA treated skin. A, D: Control skin showed normal intercellular lipid bilayers (d, arrow). B, E: UA treated skin also showed complete intercellular lipid bilayers (arrows). C, F: ONA applied skin showed complete intercellular lipid bilayers (arrows) and intact lacuna (L) (X 80,000).

Immunoblot analysis of PPAR α and keratinocyte differentiation markers expressions in HaCaT cells

It is reported the naturally-occurring free fatty acid, oleic acid, eicosatetraynotic acid, linoleic acid, and synthetic molecules, such as Clofibrate and WY 14, 643, activate PPAR α [30,31]. We finally examined whether the UA and ONA boost the PPAR α expression and sequentially enhance the keratinocyte terminal differentiation by measuring the expression level of cornified envelope associated proteins such as involucrin, loricrin, filaggrin in HaCaT cell. As seen in Fig 8, both UA (10 μM) and ONA (10 μM) induced PPAR α expression. For proof the correlation with keratinocyte terminal differentiation, we testified the levels of involucrin, loricrin, and filaggrin protein expression after 24 h incubated with (10 μM) and ONA (10 μM). As shown in Fig 8, the protein levels of involucrin, loricrin, filaggrin also increased following treatment with UA and ONA, respectively. These data indicate that the effects of UA and ONA associated to epidermal keratinocyte differentiation at least in part, via PPAR α activation in HaCaT cell.

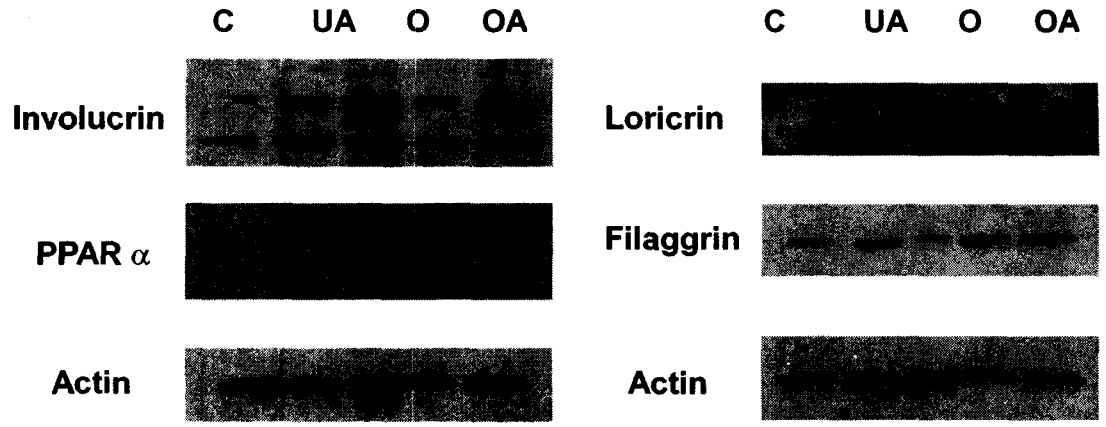


Fig. 6: Immunoblot analysis of keratinocyte differentiation markers and PPAR α in HaCaT cells treated with UA and ONA. Human keratinocytes (HaCaT) cells were stimulated with UA (10 μM) ONA(O; 10 μM), OA(200 μM) and PPAR activators Wy 14,643 (50 μM) for 24h. Whole cell lysates were prepared for immunoblot analysis. Ursolic acid (UA); UA, Oleanolic acid (ONA); O, Oleic acid; OA, Wy 14,643; Wy

Evaluate the UA and ONA effects on dermis

During the aging process, skin wrinkling occurs as a result of reductions in collagen in the dermis and epidermal atrophy [68,74]. Other fibers, elastin, maintain normal skin tension, and give it the ability to snap back after stretching. To evaluate, therefore, the effects of UA and ONA on dermis, we observed collagen fiber increasing (Fig. 6-A) and elastic fiber elongation (Fig. 6-B) using Massion-trichrome and elastic fiber staining respectively.

In vitro results of collagen and elastin synthesis and elastase inhibitory experiments were also confirmed *in vivo* results. As shown in figure 7-A, collagen was at least 85 fold higher in cells treated with 1 μM UA and ONA. However it didn't show the much effective collagen synthesis compared to Retinol treatment. Especially elastin synthesis was increased approximately 35 and 4.8 fold in cells treated with 1 μM UA compared to control and 5 μM Retinol treated group respectively but ONA didn't affect the elastin synthesis (Fig. 7-B). It was also investigated that the inhibitory effects of UA and ONA on elastase activity (Fig. 7-C). As shown in figure 7-C, UA showed inhibitory effect on elastase activity about 60 up to 80% at 20 ~ 40 μM concentration. ONA also showed inhibitory effect on elastase activity, however it is not as much effective as UA treated group.

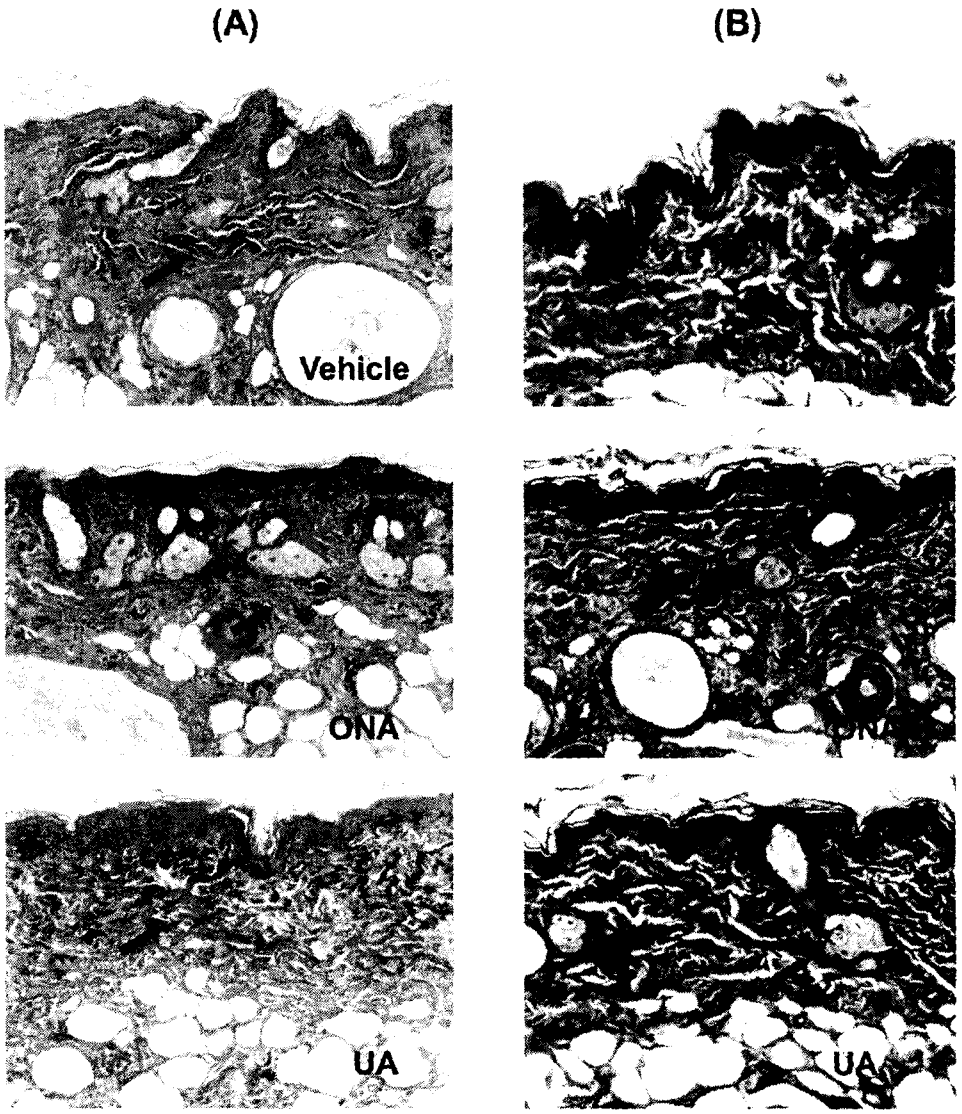
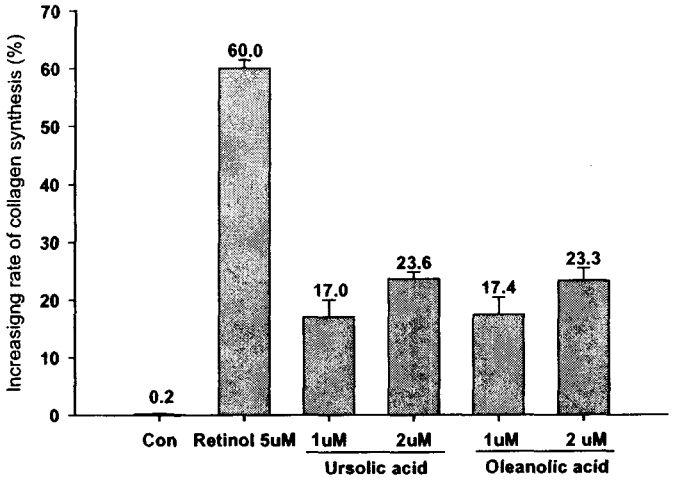
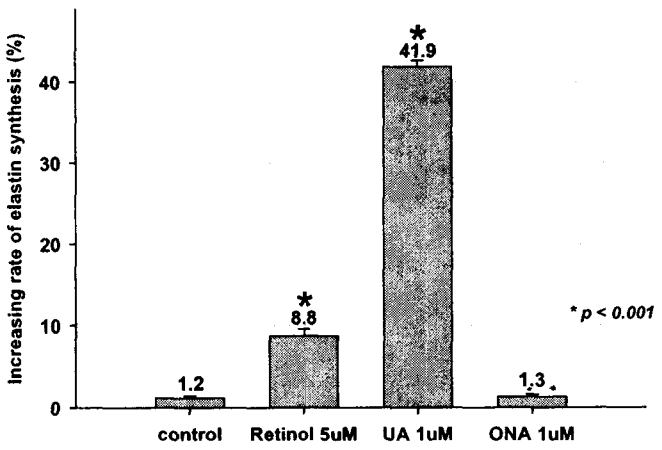


Fig. 7: Effects of UA and ONA on collagen and elastic fiber staining *in vivo*. (A) Using Masson-trichrome, UA and ONA (2mg/ml) observed collagen thickening by UA and ONA treatments (3 weeks). Especially UA 3wk treated species was showed the notable increasing and thickening of collagen (arrow; blue stained in dermis). (B) Using elastic fiber staining, UA and ONA (2mg/ml) treated observed elastic fiber increasing and elongation (arrow; dark blue stained regions in dermis) by UA and ONA treatments (3 weeks).

(A)


Increasigng rate of collagen synthesis (%)
70
60
50
40
30
20
10
0
60.0
0.2
17.0
23.6
17.4
23.3
Con
Retinol 5uM
1uM
2uM
1uM
2 uM
Ursolic acid
Oleanolic acid

(B)


Increasing rate of elastin synthesis (%)
40
30
20
10
0
*
41.9
*
8.8
1.2
1.3
* p < 0.001
control
Retinol 5uM
UA 1uM
ONA 1uM

(C)

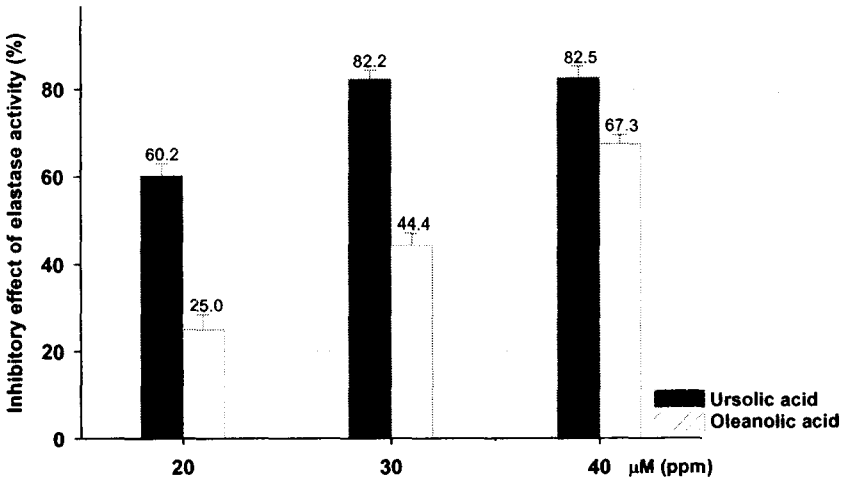

Inhibitory effect of elastase activity (%)
80
60
40
20
0
60.2
25.0
82.2
44.4
82.5
67.3
Ursolic acid
Oleanolic acid
20
30
40
μM (ppm)

Fig. 7: Effects of UA and ONA on collagen and elastin synthesis and elastase inhibition *in vitro*. Fibroblast cells were pretreated with UA and ONA at various concentration for 24h then measured collagen synthesis by the Sircol™ assay (A) and elastin synthesis by the FASTIN™ assay (B) (*See the materials and methods)*. (A) UA and ONA didn't show the difference in collagen synthesis. All parameters $p<0.005$ (B) UA increased the elastin synthesis significantly. (*p<0.001), Retinol (5 μM) was used as a positive control. (C) Porcine pancreatic elastase was assayed (*see the materials and methods*), using substrate and observed the release of p-nitroaniline. Blanks contained all the components except the enzyme.

Discussion

UA and ONA both belong to pentacyclic triterpenoid acids. They have a similar molecular structure, but they have different sites of the methyl group on the E loop: if the methyl group at C19 of UA is moved to C20, it becomes ONA. They are of interest to scientist because of their biological and pharmacological activities. UA and ONA possess antifungal [32,33], insecticidal [34], anti-HIV [35,36], diuretic [37], complement inhibitory [38], liver protection [39-42], and anti-inflammatory effects [43-46]. Recently, it was found that they had marked anti-tumor effects and exhibited cytotoxic activity toward many cancer cell line in culture [47-53]. UA and its isomer, ONA have been recommended for skin cancer therapy in Japan [54]. In this study, we have investigated the effect of UA and ONA on acute barrier disruption and normal epidermal barrier.

Impaired barrier function may be interrelated with several skin disease, possibly caused by the differences in SC lipid composition and abnormal lamellar structure of intercellular lipids. These specialized lipids, which constitute the structural components of the epidermal permeability barrier, are secreted into the extracellular space by specific cell organelles: the lamellar bodies or membrane coating granules (MCG) [55]. Sensitive skin is associated with increased TEWL, increase penetrability, and higher susceptibility to irritants. It indicates that sensitive skin is a clinical state associated with impaired barrier function. The dry and itch skin with atopic dermatitis displays impaired permeability barrier function as indicated by increased TEWL, diminished water-holding properties and decreased ceramide levels. Tape stripping eliminates not only intercellular lipids but also the cellular components of the SC. Applications of UA and ONA (0.1 mg/ml and 0.5 mg/ml each) onto the hairless mice accelerate barrier repair at 6h (Fig 2).

The skin barrier is the main structure protecting the body by separating the internal and external environments. The SC is the primary structure of the skin barrier, and has the most important functions. The SC is made up of intercellular layers of abundant hydrophobic lipid and corneocytes enveloped by the lipid layers. SC intercellular substances are composed of hydrophobic and hydrophilic layers. These lamellar structures are formed and maintained by LB that supply lipids and enzymes for the synthesis of ceramide and free fatty acid.

To confirm the effects of UA and ONA on normal skin, hydration and TEWL were measured for 1 and 3 weeks after UA and ONA treated. Hydration was increased in both UA and ONA treated group compared to vehicle from 1 week without TEWL alteration ($p<0.005$) (Fig 3, 4).

Our EM studies revealed SC layers and intercellular lipid bilayers in normal epidermal barrier with long-term treatment. There were no distinguished differences SC layers number between UA and ONA application. However, SC numbers in the skin with the UA and ONA were slightly

increased compared to vehicle (Fig 5). It is also showed well-conserved intercellular lipid bilayers and intact lacuna in UA and ONA treated groups as like vehicle group even 3 weeks application (Fig 5). It is recommend that appropriate UA and ONA treatment have no defects for epidermal barrier formation. Observation of number and secretion of LB increased slightly in UA and ONA treated group compared to vehicle (Data not shown). The appropriate LB increasing means that UA and ONA may augment the resistance of the skin barrier to irritants and, therefore, prevent dry skin.

Activators of PPAR α were reported to accelerate epidermal differentiation and barrier formation [19,21,22,24,25,56]. These results would seem to directly implicate PPAR α in the control of keratinocyte differentiation and function. Indeed, the effect of topical application of the PPAR α agonist Clofibrate on epidermal differentiation and marker gene expression was shown to be dependent on PPAR α expression [24]. The fact that the epidermis of PPAR α deficient mice appears grossly normal with only minor detectable differences in comparison with wild type mice, however, indicates that PPAR α does not play a decisive role on the control of epidermal proliferation and differentiation, or that other transcription factors may compensate for the lack of PPAR α expression [24]. Furthermore, a number of observations would seem to argue against PPAR α and γ being critical proximal mediators or executors controlling keratinocyte differentiation and function. Addition of PPAR γ ligands to keratinocytes *ex vivo* does not influence differentiation [21,22]. Synergistic activation of the functional PPAR α/retinoid x receptor (RXR) heterodimer by simultaneous administration of PPAR α and RXR ligands is a characteristic of PPAR α mediated transactivation [57-58]. Yet, no synergistic effect of PPAR α and RXR ligands on keratinocyte differentiation *per se* was observed [22]. It is known that PPAR α activators profoundly influenced lipid metabolism in reconstructed epidermis, and in this context synergy between RXR and PPAR α ligands was observed [20]. The overlapping modes of action of UA and ONA observed in this study may reflect interaction with its receptor system. It is already reported the naturally-occurring free fatty acid, oleic acid, eicosatetraynotic acid, linoleic acid like Clofibrate and WY 14, 643, activate PPAR α [30]. Evidence to date indicates that UA and ONA exert effect by binding to the PPAR nuclear receptor superfamily. Also epidermal differentiation comprises the sequential expression of keratins 1 and 10, involucrin, loricrin, filaggrin, and variety of others proteins of the CE [59]. Therefore we investigated that UA and ONA stimulate epidermal keratinocyte differentiation via PPAR α in normal human keratinocytes (HaCaT) cells. Expression levels of three keratinocyte terminal differentiation markers, involucrin, loricrin and filaggrin, increased 2 or 3 (ONA and UA, respectively) fold (Fig 7). These results suggested that the effects of UA and ONA improve not only skin barrier functions but also accelerate epidermal keratinocyte differentiation via PPAR α.

Induction of both PPAR α and PPAR γ has been linked to differentiation of human

keratinocytes [19]. In addition, synthetic analog of ONA has been shown to stimulate adipocyte differentiation via binding to PPAR γ [60]. In this study, however, UA and ONA did not induce the PPAR γ in HaCaT cells (data not shown). In comparison with the PPAR α, PPAR δ, and PPAR γ ligand binding pockets are closer in size and shape to each other. It is reported that a major determinant of selectivity between these two subtypes in the substitution of Tyr-314 in PPAR α for His-323 in PPAR γ. These amino acids form part of the network of hydrogen-bonding residues that involved in the activation of the receptor by its acidic ligands [61]. Although all three PPAR subtypes bind to polyunsaturated fatty acids with micromolar affinity, only PPAR α binds to a wide range of saturated fatty acids [62]. This property may be important for its proposed role in the regulation of hepatic lipid metabolism in response to saturated fats [62-65]. The PPAR α pocket is more lipophilic and less solvent exposed than the corresponding pockets of either PPAR γ or δ. Among the three subtypes, PPAR α may be the best suited to bind to the more lipophilic saturated fatty acids.

Induction of PPAR α also has been linked to increased synthesis of ceramides [20]. SC ceramides decrease by approximately 10-15% per decade after the age of 20 years [7,66]. Previous research showed that treatment with lotion containing 0.3% UA liposomes for 11 days increased both nonhydroxy and hydroxy ceramides [67]. Reduction in both ceramides and collagen are known to occur as a result of the aging process [66,68]. Reductions in ceramides are associated with reduced barrier function, and dry flaky skin [7,66]. In general, induction of ceramide synthesis is usually associated with keratinocyte differentiation. Induction of ceramides by UA liposomes occurs in the absence of keratinocyte differentiation. In this respect, UA liposomes exert effects on NHEK similar to those of retinoids. In the case of retinoids, reductions in these markers of differentiation in cell culture studies have been linked to epidermal thickening in skin with resultant reduction of wrinkling [69]. However, unlike retinoids, which decrease ceramide synthesis [70], UA liposomes stimulate ceramide synthesis [67,71,72]. Therefore UA liposomes may have the potential to stimulate both epidermal thickening and barrier function. As shown Fig 4, Epidermal thickness and smoothness were observed under LM for confirming epidermal changes with the UA and ONA applications ($P<0.05$). LM findings demonstrated SC was slightly increased and especially epidermal thickening (table 1) and flattening was observed (Fig. 4).

In conclusion, using UA and ONA, the effects of pentacyclic triterpene acid on skin barrier functions and correlation with PPAR α. This study demonstrates that naturally occurring UA and ONA improve skin barrier functions and stimulate epidermal differentiation and that this effect via PPAR α. It is possible that UA and ONA can be pertinent candidates to improve epidermal barrier recovery as skin therapeutic agent and preserve barrier function as cosmeseuticals.

Conclusions

1. The recovery rate increased in UA and ONA groups at 6h more than 20% compared to vehicle ($p<0.05$).

2. UA and ONA increased hydration compared to vehicle group from 1 week without TEWL alteration ($p<0.005$).

3. LM finding showed SC thickening was slightly increased and especially epidermal thickening and flattening was observed (UA>ONA>Veh).

4. EM finding revealed that secretion and numbers of lamellar bodies and complete formation of lipid bilayers were most prominent (ONA>UA>Veh).

5. Using Masson's trichrome and elastic fiber staining, we observed collagen thickening and elastic fiber increasing by UA and ONA treatments *in vivo* and UA and ONA increased the synthesis of collagen and elastin (only UA) in keratinocyte cells *in vitro*.

6. *In vitro* results of collagen and elastin synthesis and elastase inhibitory experiments were also confirmed the effects of UA and ONA on dermis. UA and ONA both affects to collagen synthesis and elastase inhibitory activity, however UA only increased the production of elastin

7. Taken together, all results suggested that the effects of UA and ONA related to not only skin barrier functions but also dermal collagen, elastic fiber synthesis and elastase inhibitory activity.

8. It is investigated that the effects of UA and ONA on keratinocyte differentiation which related to PPAR α.

In conclusion, UA and ONA can be pertinent candidates to improve epidermal permeability barrier and dermal functions and for skin therapeutic agent.

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